Intracellular Accumulation of Collagen VII in Cultured Keratinocytes from a Patient with Dominant Dystrophic Epidermolysis Bullosa

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Expression of collagen VII, a candidate molecule for dystrophic epidermolysis bullosa, was analyzed in cultured keratinocytes from a patient with generalized dominant dystrophic epidermolysis bullosa (DEBD) of the Pasini subtype. Double immunofluorescence revealed an increased intracellular staining of collagen VII that co-localized with protein disulfide isomerase, a marker of the rough endoplasmic reticulum. Ultrastructural analysis of cultured DEBD cells showed dilated cisternae of the rough endoplasmic reticulum and numerous residual bodies, both of which contained abundant collagen VII as detected by immunoelectron microscopy. Immunoblotting of keratinocyte extracts indicated an increased ratio of cell-associated versus secreted soluble collagen VII in DEBD cells. Collagen VII mRNA was of normal size in the DEBD cells, but present in excessive amounts. The data suggest a mutation in the collagen VII gene that leads to intracellular accumulation and degradation of this collagen, and thus to a reduced number of anchoring fibrils at the dermo-epidermal junction, and subsequently to blistering of the skin in this family. *Key words: anchoring fibrils/basement membrane/bullous dermatoses/connective tissue/heritable disorders/immunoelectron microscopy. J Invest Dermatol 102:105–110, 1994*

Dystrophic epidermolysis bullosa (EBD) represents a heterogeneous group of hereditary skin disorders that are characterized by blistering of the skin after minor trauma and subsequent scarring [1–3]. The cleavage occurs below the dermal-epidermal basement membrane, at the level of the anchoring fibrils that attach the epidermis to the dermal connective tissue. Ultrastructural investigations have implicated quantitative and qualitative abnormalities of the anchoring fibrils in these diseases [4–6], and immunochemical studies have suggested collagen VII, the main structural component of these fibrils, as a candidate molecule for abnormalities in EBD [7–9]. In fact, recent analyses established a linkage between the collagen VII gene on chromosome 3 and EBD in families with both dominant and recessive inheritance [10,11].

The collagen VII molecule consists of a long central triple helix, a large globular N-terminus (NC-1), and a small globular C-terminus (NC-2). Two molecules form an antiparallel tail-to-tail dimer with a small carboxy-terminal overlap and with the amino-termini pointing outwards. The dimers aggregate laterally in a non-staggered manner into the anchoring fibrils, which form a three-dimensional network between the basement membrane and the anchoring plaques in the papillary dermis [12–14].

Collagen VII is synthesized mainly by keratinocytes [15–17]. The details, however, of the transport and deposition of the molecules onto the dermal side of the basement membrane and the fibrillogenesis of the anchoring fibrils still remain unknown. Because it seems likely that abnormalities of collagen VII lead to EBD, we investigated the early events, biosynthesis, and cellular transport of collagen VII metabolism in cultured keratinocytes from a patient with dominant dystrophic epidermolysis bullosa (DEBD), a subtype in which reduced number and rudimentary structure of anchoring fibrils at the dermo-epidermal junction are characteristic features [4–6].

MATERIALS AND METHODS

Patient The family investigated here included the 43-year-old male propositus and his 10-year-old affected son. There was no consanguinity in the family, and other family members were free of skin disease. Both affected probands suffered from blistering and scarring of the skin since birth. Later, the blisters were restricted mainly to the distal extremities but could arise anywhere after mechanical trauma; blistering of the mucous membranes occurred in childhood, but ceased with advancing age. Finger and toe nails became increasingly dystrophic. Both individuals exhibited albopapuloid lesions (Pasini papules) on the lower back. The father (G.T.) was biopsied in a clinically uninvolved area on the upper thigh for morphologic analysis, and keratinocyte cultures were initiated from roofs of fresh traumatic blisters. The son (R.T.) had previously had a biopsy for diagnostic EM and refused a new biopsy.

Cell Cultures Keratinocytes were obtained from control skin or from a DEBD blister roof by trypsinization and cultured in serum-free keratinocyte growth medium as described previously [17]. Cells from the first or second passage were used in the experiments. For IF staining, electron microscopy (EM), or immunoelectron microscopy (IEM), subconfluent cells on glass or

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Abbreviations: DEBD, dominant epidermolysis bullosa dystrophica; PBSSA, phosphate-buffered saline with 0.05% saponin and 1% bovine serum albumin; PDI, protein disulfide isomerase; rER, rough endoplasmic reticulum.

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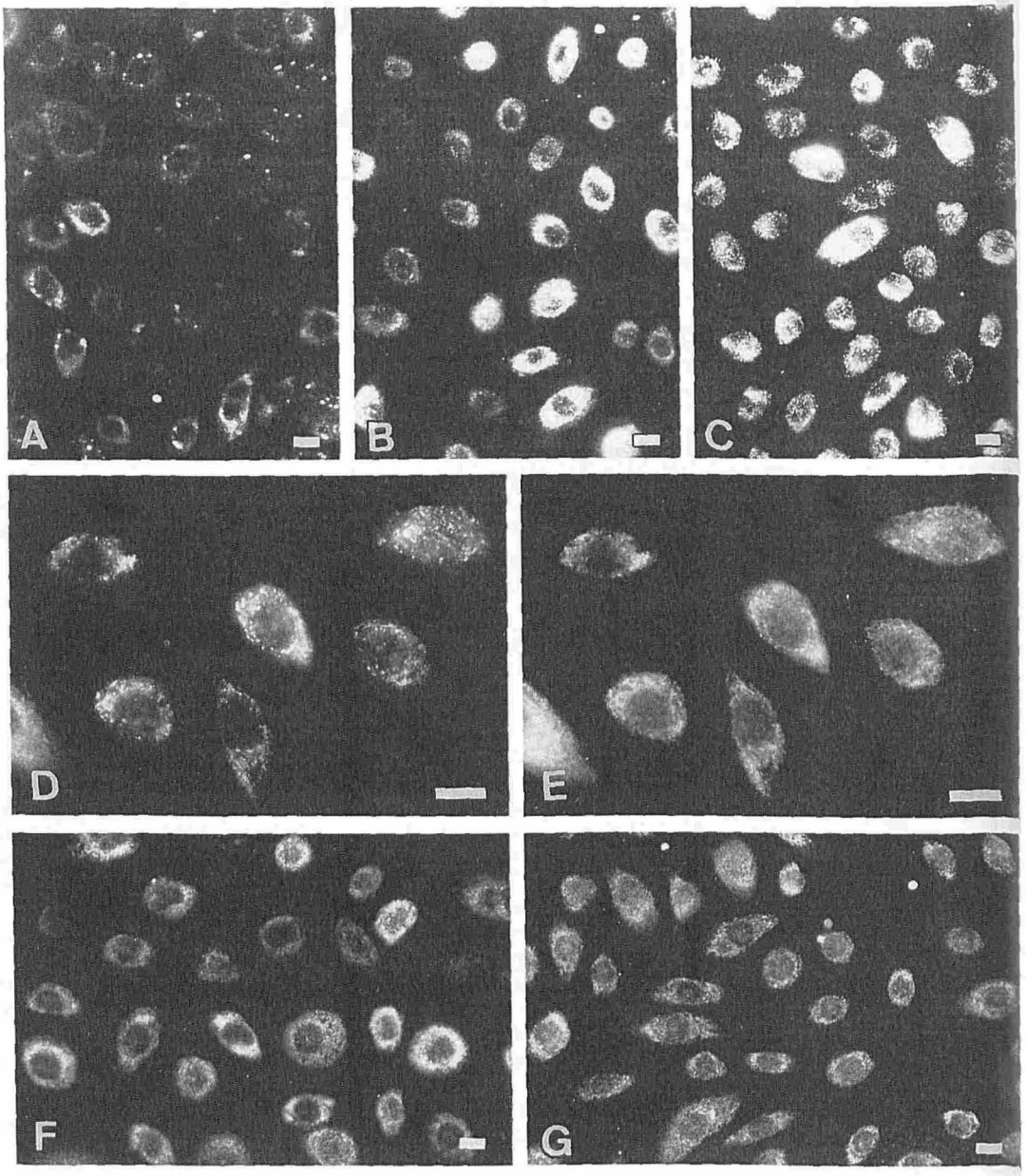

Figure 1. Expression of collagen VII and laminin in cultured keratinocytes. IF staining of normal *(A,B,F)* and DEBD *(C–E,G)* keratinocytes with antibodies to collagen VII *(A–D)*, PDI *(E)*, and laminin *(F,G)*. Collagen VII expression was low in normal cells *(A)*, but was stimulated by addition of 5 ng/ml TGF-β_2 to the medium for 48 h [17] *(B)*. A comparable strong intracellular staining was found in untreated DEBD cells *(C)*. Double-labeling IF of DEBD cells for collagen VII *(D)* and PDI, a marker for the rER *(E)*, demonstrated colocalization of both antigens. Laminin expression in normal cells *(F)* was indistinguishable from that in DEBD cells *(G)*. *Bars*, 10 μm.

plastic cover slips were used. For protein analysis, cells were grown to early confluency, and 48 h prior to protein extraction, 50 μg/ml ascorbate and in some experiments also 5 ng/ml TGF-β_2 was added into the medium [17].

Antibodies and Indirect Immunofluorescence Staining Polyclonal antibodies to the triple-helical domain of human collagen VII were affinity purified as described [18]. The monoclonal antibody LH-7.2 to the NC-1 domain of collagen VII was purchased from Chemicon International, Inc. (Temecula, CA). Polyclonal anti-human laminin antibody was a kind gift from Dr. M. Paulsson, University of Bern, Bern, Switzerland, and a monoclonal antibody to collagen IV from Dr. B. Odermatt, University of Zürich, Zürich, Switzerland. The monoclonal antibody to protein disulfide isomerase (PDI), the FITC-labeled anti-rabbit antibodies, and the peroxidase-labeled anti-mouse antibodies were purchased from Dakopatts (Glostrup, Denmark). The Texas Red-labeled polyclonal anti-mouse antibodies were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA) and peroxidase-labeled goat anti-rabbit antibodies from Kirkegaard & Perry (Gaithersburg, MD). For IF staining, subconfluent cells on cover slips were permeabilized and fixed in methanol for 15 min at −20°C and incubated with the first antibody at room temperature overnight and with the second antibody for 1 h.

Electron Microscopy Electron microscopy on clinically uninvolved skin of the patient was performed using standard techniques. For EM of cultured keratinocytes, cells were seeded on glass coverslips and grown in the presence of ascorbate to confluency. They were fixed *in situ* with 2.5% glutaraldehyde in cacodylate, pH 7.3, for 1 h at room temperature and postfixed with 2% osmium tetroxide for 30 min. The cells were dehydrated in an ascending ethanol series and directly passed from 100% ethanol to EPON 812 [19] and equilibrated overnight at 7°C. After a change of the resin, the monolayers were flat-embedded using resin-filled gelatin capsules that were positioned upside down onto the cells.

Immunoelectron Microscopy Confluent keratinocytes, grown on four-well chamber slides in the presence of ascorbate were fixed in freshly prepared 2% paraformaldehyde/0.1% glutaraldehyde/0.05% saponin in 0.1 M cacodylate, pH 7.3, for 1 h at room temperature [20]. Free aldehyde groups were quenched in 50 mM ammonium chloride in PBS/0.05% saponin (PBSSA) twice for 10 min. To block unspecific binding sites, normal goat serum diluted 1:100 in PBSSA was applied for 30 min at room temperature. Excess fluid was drained off and replaced by affinity-purified antibody to collagen VII diluted with an equal volume of double-strength PBSSA or by normal rabbit serum 1:100 in PBSSA as a negative control for 16 h at 7°C. The slides were washed three times for 10 min in PBSSA at room temperature and incubated with horseradish peroxidase–coupled (Fab)$_2$ goat-anti rabbit diluted 1:100 or 1:200 in PBSSA for 2 h. After three washes with PBSSA, the cells were post-fixed in 2.5% glutaraldehyde, washed in 7.5% sucrose, 0.05 M Tris, pH 7.4, and the diaminobenzidine reaction was carried out as described in detail [21]. The cells were then

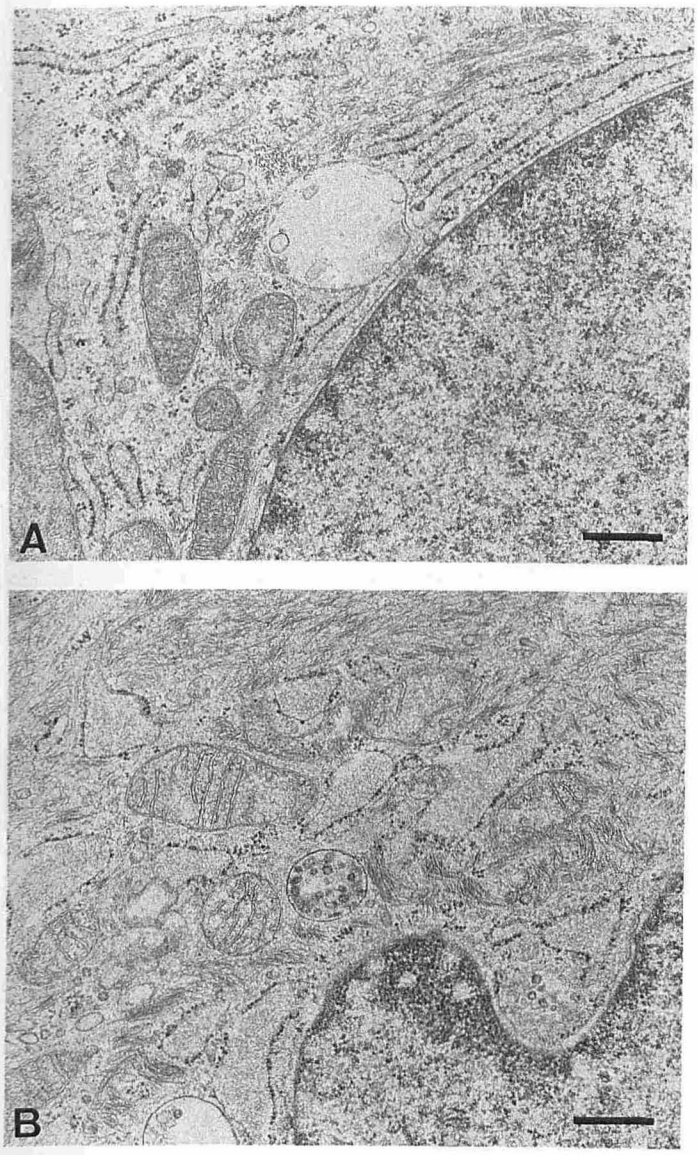

Figure 2. Electron microscopy of cultured normal and DEBD keratinocytes. *A)* In control keratinocytes, the cisternae of the rER and the perinuclear envelope are slender. *B)* In the EBD cells, the rER cisternae are dilated giving a vesicular appearance; they contain electron-dense granular material. The perinuclear envelope is unchanged. In both cultures, tonofilaments and cytoplasmic vacuoles are typical features. Counterstained with lead citrate and uranyl acetate. *Bars,* 1 μm.

osmicated and embedded as described above. Only lead citrate counterstain was used.

Collagen VII Extraction and Immunoblotting Keratinocytes in early confluency were incubated with 50 μg/ml ascorbate for 48 h and the cell layer and the medium were extracted as described elsewhere [17]. The aminoterminus of collagen VII was isolated from ethanol-precipitated keratinocyte extracts. The pellets were suspended in a buffer containing 0.15 M NaCl, 5 mM calcium chloride, 50 mM Tris-HCl (pH 7.4), and 10 mM NEM [22]. Purified bacterial collagenase (CLSPA with 643 units/mg; Worthington Biochemical Corp., Freehold, NJ) was added to a final concentration of 10 μg/ml. After incubation for 4 h at 39°C, the digestion was stopped by boiling in 2% SDS for 5 min, and the samples were dialyzed against SDS-PAGE sample buffer.

The extracts were separated by SDS-PAGE with a 4.5–15% gradient [23] under reducing conditions and transferred onto nitrocellulose [24] in the presence of 0.1% SDS. After blocking with 2% defatted milk powder in TBS for 30 min, the nitrocellulose sheet was incubated with antibodies to collagen VII overnight, followed by peroxidase-labeled second antibodies for 2 h, and 4-chloro-1-naphtol as chromogen.

Northern Blotting Poly A+ RNA was isolated using a Pharmacia QuickPrep mRNA Purification Kit (Pharmacia LKB Biotechnology Div., Dubendorf, Switzerland) and separated on a 1% agarose gel containing 2.2 M formaldehyde, transferred onto a Nytran nylon filter (Schleicher & Schuell, Dassel, Germany) and immobilized with UV-linking (Stratalinker, Stratagene, Heidelberg, Germany). The filters were prehybridized and hybridized [25] with the 1.9-kb K131 collagen VII cDNA [26], which was radiolabeled with ^{32}P-dCTP using Pharmacia Oligolabelling Kit (Pharmacia LKB Biotechnology Div., Dubendorf, Switzerland). A cDNA of GAPDH, a ubiquitous gene, was used as reference probe to correct for differences in RNA loading [27].

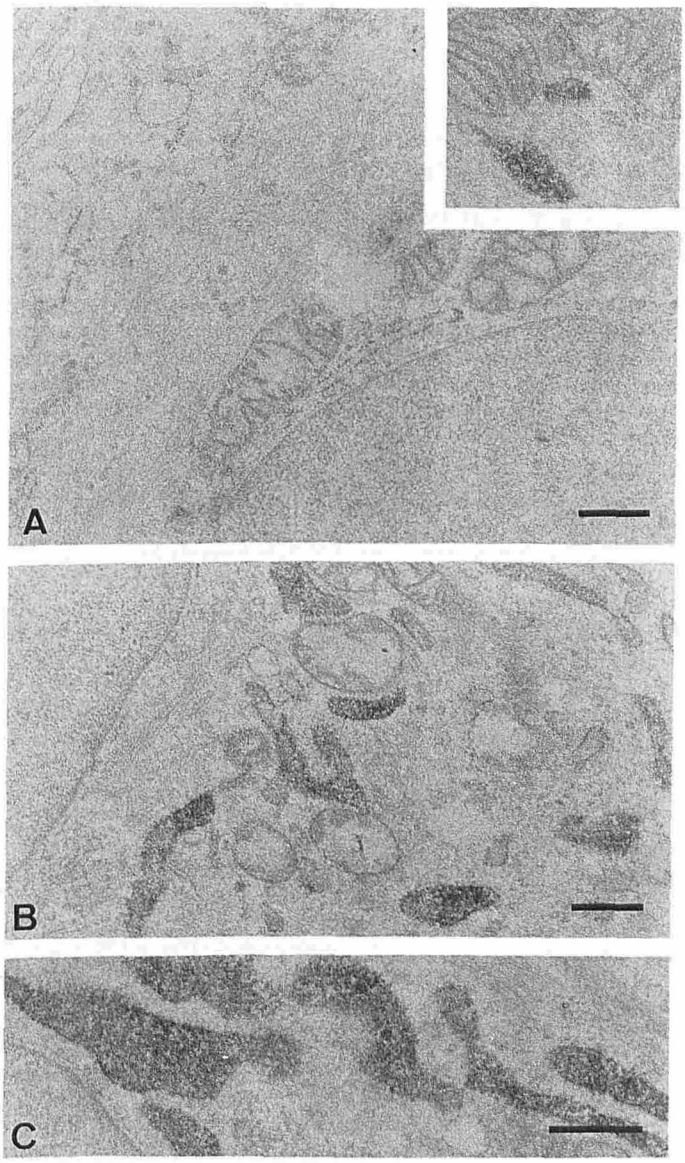

Figure 3. Pre-embedding immunoelectron microscopy of cultured control and DEBD keratinocytes. *A)* In control cells, cisternae of the rER were mostly negative for collagen VII. Occasionally some rER profiles contained the antigen *(inset)*. The upper left corner shows several digitiform processi in the periphery of the cell. *B)* In the DEBD cells, numerous dilated rER cisternae contain the granular immunoperoxidase reaction product indicative of accumulated collagen VII in the luminal space. *C)* At higher magnification, the ribosome studding of dilated rER cisternae becomes visible. The perinuclear envelope is free of antigen. Lead citrate counterstain only. *Bars,* 0.5 μm.

RESULTS

Collagen VII Expression in Skin Control skin showed a normal dermo-epidermal junction with numerous cross-banded anchoring fibrils with frayed ends emerging from the lamina densa into the papillary connective tissue. Clinically uninvolved DEBD skin exhibited focal dermo-epidermal splitting below the basement membrane, besides extended stretches of unseparated junction zone. In all areas anchoring fibrils were reduced in number and were partially missing. Most of them appeared normal, with the typical cross-striation pattern, but some were rudimentary. The basement membrane and the hemidesmosomes appeared normal. There was no difference in the appearance of the rough endoplasmic reticulum of the basal keratinocytes in control and DEBD epidermis (not shown). IF staining with antibodies to laminin, collagen IV, and collagen VII showed a linear fluorescence at the blister roof, thus confirming cleavage below the lamina densa and the diagnosis of EBD [2,3,9]. Antibodies to both the triple-helical and the globular N-terminal domain of collagen VII produced a linear staining along the basement membrane at the blister roof, but no staining at the blister base. Notably, no intracellular staining of collagen VII in the epidermis was evident (not shown).

Collagen VII Expression in Cultured Keratinocytes In contrast to normal keratinocytes that usually give a weak IF signal for collagen VII (Fig 1*A*), DEBD cells exhibited a strong intracellular staining (Fig 1*C*) comparable to that of normal cells stimulated with TGF-β_2 [17] (Fig 1*B*). Double IF staining with antibodies to collagen VII (Fig 1*D*) and to PDI (Fig 1*E*), a marker of the rough endoplasmic reticulum (rER) [28,29] revealed co-localization of the two proteins. IF staining with antibodies to laminin showed a normal fluorescence pattern in both control (Fig 1*F*) and DEBD keratinocytes (Fig 1*G*). Negative controls with rabbit and mouse preimmune sera displayed no staining.

Ultrastructure of Keratinocytes Cultured control cells appeared round and with many digitiform processes. A characteristic feature was the presence of many vacuoles as already visible by phase-contrast microscopy, and abundant tonofilaments. The cells were rich in mitochondria and displayed numerous slender cisternae of the rER (Fig 2*A*). In contrast, the DEBD cells showed dilated rER cisternae of vesicular appearance, filled with an electron-dense content (Fig 2*B*). In addition, myelin figures (residual bodies) were prominent. These ultrastructurally defined structures are thought to represent remnants of autophagocytotic processes [30]. There was no sign for an alteration of the Golgi apparatus and associated vesicles. In control cells, most of the slender rER cisternae were unlabeled (Fig 3*A*), and the granular immunoperoxidase product was found only occasionally in some rER profiles (inset of Fig 3*A*). In contrast, the DEBD cells showed far more immunoreactive rER profiles, and the content of the dilated cisternae was strongly stained (Fig 3*B,C*). Immunoperoxidase staining was also found in many myelin figures (Fig 4*A–C*). In both cell cultures, Golgi cisternae and Golgi-derived vesicles were negative for collagen VII (Fig 4*D*). Negative control with rabbit preimmune showed no immunolabeling.

Immunoblotting of Keratinocyte Extracts Collagen VII was detectable in medium and cell layer of normal and DEBD cells (Fig 5*A*). However, a clearly increased amount of collagen VII was present in all DEBD cell extracts as compared to controls. No obvious difference in the amount was seen in the media, indicating an increased ratio of cell-associated versus secreted soluble collagen VII in DEBD cells. In some immunoblots, the collagen VII from EBD cells appeared as a fuzzy band, with a slightly reduced mobility. To study further the structure of collagen VII from DEBD cells, the extracts were digested with bacterial collagenase to yield the noncollagenous aminoterminal domain. As shown in Fig 5*B*, the collagenase-resistant aminoterminus from normal and DEBD cells had the same electrophoretic mobility, excluding a gross abnormality in this domain.

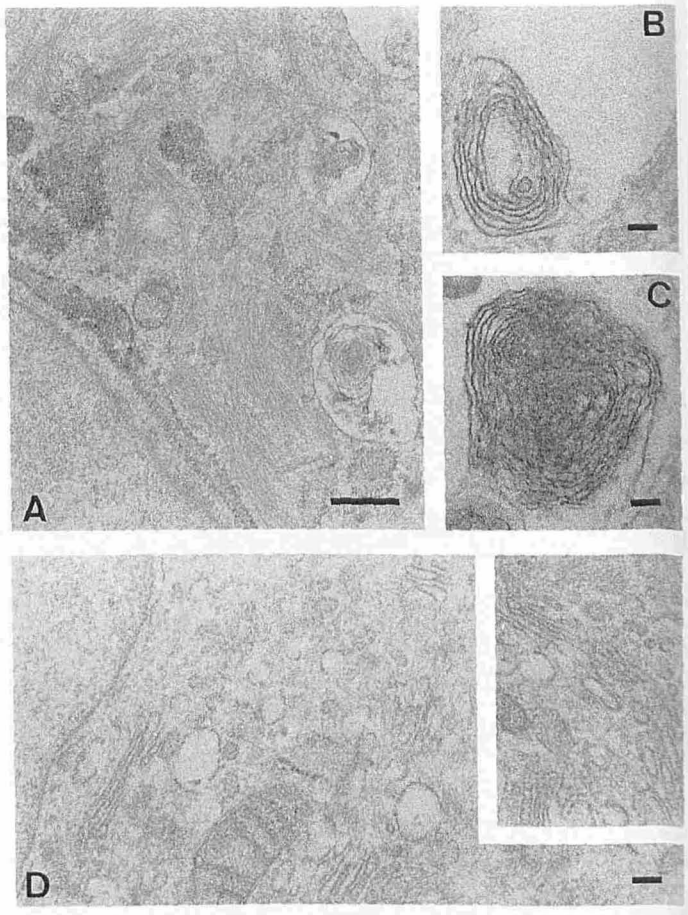

Figure 4. Involvement of other organelles in the intracellular accumulation of collagen VII in DEBD as revealed by IEM. *A)* A characteristic feature of EBD cells was the prominence of myelin figures (residual bodies). *Bar*, 1 μm. *B)* Some of them were negative for collagen VII. *C)* Other myelin figures were heavily labeled for collagen VII. *D)* Whenever found in a section, the Golgi apparatus and associated vesicles were negative for collagen VII in both control *(inset)* and EBD cells. Lead citrate counterstain only. *Bars* in *B–D*, 0.1 μm.

Northern Blotting Northern blots of mRNA isolated from DEBD cells and controls were hybridized with radiolabeled K131, a 1.9-kb cDNA of collagen VII that spans the transition of the aminoterminal to the triple-helical domain [26]. The blots demonstrated the same, single mRNA species of about 9 kb in both cells (Fig 6), but DEBD cells exhibited a stronger signal than controls.

DISCUSSION

We provide evidence for intracellular retention of collagen VII in the DEBD patient's cultured keratinocytes. The collagen accumulated within the rER of the DEBD cells and led to dilated cisternae. In addition, it was localized in residual bodies, indicating its intracellular degradation by autophagocytosis. The retention was not due to a generally impaired secretion in DEBD keratinocytes, because no accumulation of other basement membrane components, like laminin or collagen IV, was observed. Interestingly, no collagen VII was detected in the epidermal keratinocytes in the patient's skin. This may not, however, be surprising because also under normal circumstances intracellular collagen VII cannot be visualized in adult skin, probably because it is produced in minimal amounts during a normal steady state with a low turnover. This is different in situations with increased matrix synthesis: intracellular collagen VII or its mRNA were found in fetal skin or during wound healing [31,32]; Zambruno G, Cavani A, Giannetti A, Bruckner-Tuderman

L: *J Invest Dermatol* 98:529, 1992 [abstr]. Detectable amounts of collagen VII have been also observed *in situ* in the epidermis of infants with transient bullous dermatosis of childhood, a rare childhood blistering disease of short duration and of unknown etiology [33] and in some newborns and infants with EBD. Light [33–35] and electron microscopic [34] studies of the skin revealed unusual vesicles in epidermal keratinocytes, the content of which stained with antibodies to collagen VII. However, it is not clear whether this intraepidermal staining was solely due to the disease or also reflected the developmental stage of the skin in these very young patients. No data on continued intraepidermal collagen VII expression during the course of the disease or on collagen VII expression *in vitro* in cultured keratinocytes of these patients have been reported.

The increased levels of mRNA and synthesis of collagen VII observed here point to impaired feedback inhibition, analogous to that observed for collagen I in fibroblasts derived from dermatosparaxis in man and animals (for review, see [36]). The normal size mRNA, together with the slightly slower electrophoretic mobility of the α1(VII) polypeptide chain, suggests an abnormality of collagen VII that leads to post-translational overmodification of the molecule. Such phenomena have been described in other heritable collagen diseases, such as osteogenesis imperfecta [37] or the Ehlers-Danlos syndrome IV [36] with mutations in collagen I or III, respectively [38]. In these diseases the mutant amino acid usually resides in the triple-helical or the C-terminal globular domain, rather than in the non-collagenous amino-terminus. Therefore, it seems likely that a mutation in the collagen VII gene of the present propositus lies in the area of the gene that codes for the collagenous or the NC-2 domain. In fact, we have analyzed the cDNA sequence covering most of the NC-1 domain and the transition into the triple-helical domain [39,40] using reverse-transcriptase PCR of the patient's fibroblast mRNA, followed by SSCP electrophoresis [41], and have found no aberrant fragments indicative of mutations, suggesting that in this patient the NC-1 domain is normal (U. Kalinke and L. Bruckner-Tuderman, unpublished).

Figure 6. Northern blotting of collagen VII mRNA from normal and EB cells. The hybridization with the collagen VII cDNA K131 [26] revealed a single 9-kb collagen VII mRNA in all lanes. The signal was stronger in EBD cells than in controls. The same filter was rehybridized with a cDNA for GAPDH as a reference probe (bottom).

In analogy to what is currently known of collagen I and III in other heritable connective tissue disorders [36–38], it is likely that a mutation leads to delayed and faulty folding of three α1(VII)-chains into a collagen triple helix and thus to intracellular accumulation and degradation of this molecule. Consequently, only a small fraction of the triple helices containing one or more mutated α1(VII) chains leaves the rER. Theoretically, in a condition with equal synthesis of one normal and one abnormal allelic product, only one of eight of the homotrimeric collagen VII molecules consist exclusively of normal α1(VII) chains. The remaining seven of eight contain either one, two, or three mutant chains. If secreted, the abnormal molecules may interfere with polymerization of the anchoring fibrils or render them more susceptible to proteolytic degradation. This finally leads to reduced number, rudimentary appearance, and functional deficiency of the anchoring fibrils as characteristics for DEBD of Pasini type.

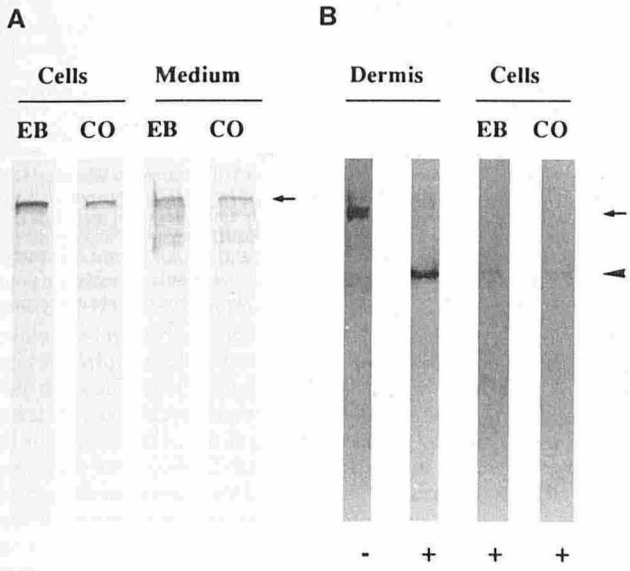

Figure 5. Immunoblotting of collagen VII from keratinocyte cultures. The cells and the medium of a confluent 25-cm² keratinocyte culture were extracted separately. *A)* Antibodies to the triple-helical domain of collagen VII. Cells: DEBD cell layer *(left)* and control cell layer *(right)*. The volume loaded corresponds to one tenth of the total culture. Medium: medium of DEBD cells *(left)* and of normal cells *(right)*. The volume loaded corresponds to one quarter of total medium. Note the higher content of collagen VII in DEBD cells. *B)* Monoclonal antibody to the aminoterminus of collagen VII. The cell extracts were prepared as described in *Materials and Methods* and incubated with (+) or without (−) purified bacterial collagenase. Dermis: collagen VII in undigested extract *(left)* and the enzyme-resistent N-terminus in collagenase-digested extract *(right)*. Cells: collagenase-digested extract of EBD keratinocytes *(left)* and controls *(right)*. The N-terminus of collagen VII showed the same electrophoretic mobility in all lanes. *Arrow,* migration position of collagen VII, $M_r \approx 250$ kD; *arrowhead,* position of the N-terminus, $M_r \approx 150$ kD.

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